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Studies on some cytokines, CD4, iron status, hepcidin and some haematological parameters in pulmonary tuberculosis patients based on duration of treatment in Southeast, Nigeria

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Abstract

The study was done to determine the levels of interferon-gamma, Interleukin 6 (IL-6), Interleukin 10 (IL-10), iron status, hepcidin and hematological parameters of patients with pulmonary tuberculosis based on duration of treatment in Southeast, Nigeria**.** This study was carried out at the Federal Medical Centre, Umuahia. A total of two hundred (200) subjects aged 18-60 years comprising of 50 subjects each for TB on baseline, on two months treatment, four months treatment and six months treatment were recruited for this study. Data were analyzed using Statistical Package for Social Science (SPSS) version 20. ANOVA (Analysis of Variance) was the tool employed. The results showed difference that was statistically significant in IFN-γ (p = 0.000), IL-6 (p = 0.000) IL-10 (p = 0.000), CD4 (p = 0.000), hepcidin (p = 0.000), Iron (p = 0.000), TIBC (p = 0.000), %TSA (p = 0.000) WBC (p = 0.000), Neutrophils (p = 0.000), Lymphocytes (p = 0.000), Monocytes (p = 0.000), Eosinophils p = 0.000), Basophils (p = 0.018), RBC (p = 0.000), Haemoglobin (p = 0.000), PCV (p = 0.000), MCV (p = 0.000), MCH (p = 0.000), MCHC (p = 0.000), Platelets (p = 0.000), ESR (p = 0.000) when compared among PTB on Baseline, two months, four months and six months of treatment respectively. The study shows that interferon gamma, IL-6, IL-10 and hepcidin are adjunct to biomarkers in the pathogenesis of pulmonary TB. The haemtological parameters like haemoglobin, RBC and PCV increased significantly with increased duration of treatment showing improvement in health status of the patients and monocytes decreased significantly in pulmonary TB patients.

Keywords: *Cytokines, CD4, Iron status, Hepcidin, Haematological paramters, Pulmonary tuberculosis patients, Duration of treatment*

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1. Introduction

Tuberculosis (TB) is a chronic bacterial disease caused by Mycobacterium tuberculosis (MTB) complex which commonly affects the lungs; (pulmonary tuberculosisTB (PTB)), but can affect other sites as well; (extrapulmonary tuberculosis (EPTB)) as opined by Thumamo *et al.* (2012). MTB, the bacterium that causes human PTB, is an old enemy. Historically; PTB has a lineage that could be traced to the earliest history of mankind having been in existence since 150,000-200,000 years ago (Okonkwo *et al.*, 2013). It is believed that PTB first made its ravaging presence felt in Europe and later got to the US, Africa and Asia through voyagers and early settlers (Okonkwo *et al.*, 2013).

PTB is a global public health problem and is the second leading cause of death. All inclusive, the disease takes a life every 20 sec (Divangahi, 2013; and Yang *et al.*, 2015). Pai and Schito (2015) reported that although much progress has been made with regard to the control measures, the World Health Organization (WHO, 2013) estimated that nine million people developed tuberculosis in 2013 and that 1.5 million deceased, including 360,000 people who were infected with human immunodeficiency virus (Pai and Schito, 2015). PTB is a major public health problem in Nigeria with an estimated prevalence of 616 cases per 100,000. Nigeria ranks first in Africa, and fourth among the 22 high pulmonary TB burden countries in the world, and no fewer than 460,000 cases of pulmonary TB are reported annually in Nigeria (WHO, 2008). Itah and Udofia (2005) reported the prevalence rate of 38.5% pulmonary TB in Ikot Ekpene and 17.6% in Itu local government area of Akwa Ibom state; they reported that male subjects had a higher incidence rate of pulmonary TB (35.6%) compared to 29.6% in female. Similarly, Nwanta *et al*. (2011) reported an overall prevalence rate of 37.9% pulmonary TB in Enugu state, Nigeria.

There are few reports on host iron status at the time of PTB diagnosis (Gangaidzo *et al.*, 2001). Friis *et al.* (2009) in their study reported iron limited erythropoiesis and anemia of inflammation during infections. The alarming increase in the incidence of PTB in our country has been made worse by elevated occurrence of HIV/AIDS (WHO, 2013). Cytokines are important immunomodulating agents of immune system.

Peptide hepcidin, is a key iron-regulatory hormone (Nemeth and Ganz, 2006), which is released from hepatocytes in response to inflammation via iron and oxygen. Interestingly, inflammation induces hepcidin production, mediated by the inflammatory cytokine IL-6. This results in sequestration of Fe in the stores and Fe-limited erythropoiesis, and eventually anemia of inflammation (Nemeth and Ganz, 2006). The study will determine hepicidin level and Interleukin 6 (IL-6). IL-6 is a proinflammatory cytokine that regulates various physiological processes (Tanaka and Kishimoto, 2014). It plays a key role in the acute phase response and in the transition from acute to chronic inflammation (Kaplanski *et al.*, 2003). Evidence has accrued to suggest that dysregulation of IL-6 production is a major contributor to the pathogenesis of chronic inflammatory diseases (Tanaka and Kishimoto, 2014; and Atreya *et al.*, 2000). This study will find out the changes that may be associated to the IL-6 levels in PTB patients. IL-6 is known to exhibit multifactorial function. It will be important to determine the changes the PTB could cause to this cytokine. The lack of reliable biomarkers to indicate or predict the different clinical outcomes of *M. tuberculosis* infection has been given as a key reason for the failure of developing new diagnostic and prognostic tolls, drugs and vaccines against tuberculosis (Mihret and Abebe, 2013).

A research by Akpan *et al.* (2012) agreed that the mean of the total WBC count in PTB patients is usually normal or not significantly elevated as compared to the normal mean of a population. This study determined the levels of hematological parameters at the point of diagnosis and during treatment.

Anemia is a major challenge in patients with PTB patients. IL-6 is known to be a major cytokine that regulates the expression of acute reactant protein, hepcidin which regulates iron level in the body; has a strong relationship with anemia and inverse relationship with CD4. Other cytokines that have not been studied on their association to hepcidin and iron status like IL-10 and IFN-γ will be studied at the point of diagnosis will be determined. In this study, iron status was determined to find out the impact of PTB on iron status which may have a role in the pathogenesis of the infection. The study will determine hepicidin level and IL-6.

1.1. Aim

The study was done to determine the levels of interferon-gamma, IL-6, Interleukin 10 (IL-10), iron status, hepcidin and hematological parameters of patients with PTB coinfected with human immunodeficiency virus on chemotherapy in Umuahia.

2. Materials and methods

2.1. Study area

This study was carried out at the directly observed treatment-short course Tuberculosis (TB DOTS) center of Federal Medical Centre, Umuahia, located in South-Eastern Nigeria.

2.2. Advocacy, mobilization, pre-survey contacts and ethical considerations

With a well detailed research proposal and a letter of introduction from the Head of Department, Consent form and an application letter were submitted to the Head, Health Research and Ethics Committee of the Institution was met. After their meetings and thorough perusal of the protocols of the research, an ethical approval was given for the study.

2.3. Study population and enrolments

A total of two hundred (200) subjects aged 18-60 years were enlisted for this study. The participants were recruited by purposive sampling technique. The PTB subjects were recruited from the tuberculosis directly observed treatment, short course (TB-DOTS) clinic based on sputum smear acid fast bacilli by Ziehl Neelsen's stain and GeneXpert MTB/RIF assay, while apparently healthy age and sex matched subjects were recruited as controls. The subjects were grouped into:

Group A: naïve PTB subjects (n = 50 subjects on baseline),

Group B: PTB subjects on two months on isoniazid, rifampicin, pyrazinamide and ethambutol treatment therapy (n = 50 subjects),

Group C: PTB subjects on four months isoniazid and rifampicin therapy (n = 50 subjects),

Group D: PTB subjects on six months isoniazid and rifampicin therapy (n = 50 subjects).

2.4. Selection criteria

2.4.1. Inclusion criteria

(i) Subjects of both sexes aged 18-60 years positive for *MTB* screening and confirmatory tests were included in the study.

(ii) Those that gave consent were included.

2.4.2. Exclusion criteria

The following subjects were excluded

a. Those that tested negative for PTB and HIV
b. Pregnant women
c. Diabetes mellitus patients
d. Persons below 18 years and above 60 years
e. Those that did not give consent.

2.5. Sample collection

Seven milliliters (7 ml) of venous blood was collected from each subject and 2.5 ml was dispensed into bottles containing di-potassium salt of ethylenediamine tetra-acetic acid (K_2-EDTA) at a concentration of 1.5 mg/ml of blood and was used for full blood count, CD4 count.

Also, 4.5 ml was dispensed into plain tubes. Serum was obtained after clotting by spinning at 3,000 RPM for 10 min and was used for interferon gamma, IL-6, and IL-10, iron and hepcidin determination.

Three separate sputum samples (consisting of one early morning sample and two spot samples) were collected in a wide mouth container from the subjects for PTB diagnosis.

The whole samples was analyzed in Links Laboratory, Owerri by Sandwich ELISA method for interferon gamma, ILs (6 and 10) and hepcidin and CD4 count, full blood count analyzed in the Diagnostic Laboratory Unit, University Health Services Department of Michael Okpara University of Agriculture, Umudike, Abia state. Ziel Nelson and GeneXpert were done in Federal Medical Centre, Umuahia, Abia state, Nigeria.

2.6. Laboratory procedures

All reagents were commercially purchased and the manufacturer's standard operating procedures were strictly adhered to.

2.7. Determinations

2.7.1. Ziehl-Nelson Technique for Mycobacterium tuberculosis diagnosis (Cheesbrough, 2006)

2.7.1.1. Smear preparation: A piece of clean stick was used to transfer and spread sputum materials evenly covering an area of about 15-20 mm diameter on a glass slide. The smear was air dried and labeled.

2.7.1.2. Heat fixation: The slide with the smear uppermost was rapidly passed three times through the flame of a Bunsen burner and was allowed to cool.

2.7.1.3. Ziehl-Nelson Staining: The slide containing the smear was placed on a slide rack and the smear covered with carbol fuschin stain. The stain was heated until vapor just begins to rise. The heated stain was allowed to remain on the slide for 5 min. The stain was washed off with clean water and then covered with 3% v / v acid alcohol for 5 min or until smear is sufficiently decolorized, that is pale pink. The slide was washed off with clean water. The smear was covered with Methylene blue stain for 2 min and then washed off with clean water. The back of the slide was wiped clean and placed in a draining rack for the smear to air dry.

2.7.1.4. *Mycobacterium tuberculosis* diagnosis: The smear was examined microscopically using the X100 oil immersion objective. Scanning of the smear was done systematically and when any definite red bacillus is seen, it was reported as AFB positive.

2.7.2. GeneXpert method for detection of Mycobacterium tuberculosis *and Rifampicin resistance (GeneXpert MTB/ FIF)*

The assay consists of a single-use multi-chambered plastic cartridge preloaded with the liquid buffers and lyophilized reagent beads necessary for sample processing.

2.7.2.1. DNA extraction and hemi-nested real-time PCR: Sputum samples were treated with the sample reagent (containing NaOH and isopropanol). The sample reagent was added in the ratio of 2:1 to the sputum sample and the closed specimen container was manually agitated twice during 15 min of incubation at room temperature. 2 ml of the treated sample was transferred into the cartridge, the cartridge was loaded into the GeneXpert instrument and automatic step completed the remaining assay steps.

The assay cartridge also contained lyophilized *Bacillus globigii* spores which served as an internal sample processing step and the resulting *B.globigii* DNA was amplified during PCR step. The standard user interface indicates the presence or absence of *MTB*, the presence or absence of rifampicin resistance and semi-quantitative estimate of *MTB* concentration (high, medium, low and very low). Assays that are negative for *MTB* and also negative for *B.globigii* internal control was reported as invalid.

2.7.3. Determination of CD4 count by flowcytometery (Partec Cyflow counter), Germany

All required reagents was brought to room temperature and 850 µl of the count check bead green will be analyzed to ensure that the cyflow machine is working properly. The desired numbers of rohren test tubes was placed in a test tube rack. 20 µl of CD4 easy count kits (CD4 Mab-PE) were pipetted into different test tubes labeled appropriately for the assay. Then, 20 µl of blood sample was also pipette into each respective test tube and incubated in the dark for 15 min at room temperature after mixing properly. This was followed by the addition of 850 µl easy count. No lyse buffer was added to each test tube. This was mixed properly to avoid air bubbles and analyzed on the Partec Cyflow. The result was displayed and copied from the screen.

2.7.4. Full blood count by automation using Mindray BC-5300, China

The sample is EDTA bottle was placed in the spiral mixer and allowed to mix very well. Whole blood mode was activated in the LCD screen, the sample no (code) was inputted via key board and then the key will be selected. Then the sample was mixed very well again and the cap was removed and inserted into the probe and the SART button was pressed. When the LCD screen displays ANALYZING; the sample was removed and recapped. The analyzer was executed automatic analysis and displays the result on LCD screen.

2.7.5. Determination of serum iron concentration by Ferozine method Teco Diagnostics (Iron/TIBC) Laketiew Ave, Acashein, CA 92807

Iron free clean tubes were labeled as test, blank and standard. The 2.5 ml of iron buffer reagent was added to all the labeled tubes. Also, 0.5 ml of the samples was added to the respective tubes and was mixed. The reagent blank was used to zero the spectrophotometer at 560 nm. The absorbance of all tubes was read and value will be recorded (A1 reading). Then, 0.5 ml of iron reagent was added to all the tubes and was mixed properly. The tubes were placed in a heating bath at 37 °C for 10 min. The reagent blank was also used to zero the spectrophotometer at 560 nm and another absorbance of all the tubes was read and the value obtained was recorded (A2 reading).

Calculation

Serum iron (μg / dl) = A2 Test-A1 Test $\times$ Con of A2 std-A1 std

where A1 Test = Absorbance of first reading of the test

A2 = Absorbance of the second reading of the test

A1 std = Absorbance of the first reading of the standard

A2 std = Absorbance of the second reading of the standard

2.7.6. Determination of total iron binding capacityby Ferozine method of TECO diagnostics (Iron/TIBC) Laketiew Ave, Acashein, CA 92807

Iron free clean test tubes were labeled as test, blank and standard and 0.2 ml of unsaturated iron binding capacity buffer reagent was added to all the tubes according to the sample number, while 10 ml of iron free water was added to standard tube and was properly mixed. To the test 0.5 ml of sample and 0.5 ml iron standard were added to the test, and was properly mixed. The reagent blank was used to zero the spectrophotometer at 560 nm wavelength. The absorbance of the samples was read and recorded as A1 reading. Also 0.5 ml iron standard tube and was properly mixed. To the test, 0.5 ml of sample and 0.5 ml iron standard were added to the test, and was properly mixed. The reagent blank was used to zero the spectrophotometer at 560 nm wavelength. The absorbance of the samples was added to the tubes and was mixed properly and was placed in a heating bath at 37 °C for 10 min. The reagent blank was used to zero the spectrophotometer at 560 nm and another reading was taken as the A2 reading.

Calculation

$$\text{UIBC } (\mu g / dl) = \frac{\text{Conc. of std} - \text{A2 Test-A1 Test}}{\text{A2 std} - \text{A1 std}} \times \text{Conc. of std}$$

TIBC (μg / dl) = Iron + UIBC

where A1 Test = Absorbance of first reading of the test

A2 = Absorbance of the second reading of the test

A1 std = Absorbance of the first reading of the standard

A2 std = Absorbance of the second reading of the standard

*2.7.7. Human Interferon-gamma (IFN-*g*) ELISA Kit by Melsin Medical Co Limited, Catalogue Number: EKHU-0162*

Dilutions of standard was prepared to get a concentration of 240 ng / l, 160 ng / l, 80 ng / l, 40 ng / l and 20 ng / l. 50 μl of standards were pipette into the standard wells. 10 μ of test serum were added into each well. 40 μl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50 μl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 min at 37 °C. It was washed for four times. 50 μl of chromogen solution A and 50 μl of chromogen solution B was added to each well. They were mixed and incubated for 10 min at 37 °C. 50 μl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450 nm wavelength within 15 min taking the blank well as zero concentration.

Calculation

A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

2.7.8. Human IL-6 commercial ELISA Kit by MELSIN Medical Co Limited was used. Catalogue Number: EKHU-0140

Dilutions of standard was prepared to get a concentration of 240 ng / l, 160 ng / l, 80 ng / l, 40 ng / l and 20 ng / l. 50 μl of standards were pipette into the standard wells. 10 μ of test serum were added into each well. 40 μl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50 μl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 min at 37 °C. It was washed for four times. 50 μl of chromogen solution A and 50 μl of chromogen solution B was added to each well. They were mixed and incubated for 10 min at 37 °C. 50 μl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450 nm wavelength within 15 min taking the blank well as zero concentration.

Calculation

A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

2.7.9. Human IL-10 Assay by commercial ELISA Kit by MELSIN Medical Co Limited was used. Catalogue Number: EKHU-0155

Dilutions of standard was prepared to get a concentration of 240 ng / l, 160 ng / l, 80 ng / l, 40 ng / l and 20 ng / l. 50 μl of standards were pipette into the standard wells. 10 μ of test serum were added into each well. 40 μl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50 μl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 min at 37 °C. It was washed for four times. 50 μl of chromogen solution A and 50 μl of chromogen solution B was added to each well. They were mixed and incubated for 10 min at 37 °C. 50 μl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450 nm wavelength within 15 min taking the blank well as zero concentration.

Calculation

A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

2.7.10. Human Hepcidin (Hepc) ELISA Kit by MELSIN Medical Co Limited was used with Catalogue Number: EKHU-1674

Dilutions of standard was prepared to get a concentration of 240 ng / l, 160 ng / l, 80 ng / l, 40 ng / l and 20 ng / l. 50 μl of standards were pipette into the standard wells. 10 μ of test serum were added into each well. 40 μl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50 μl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 min at 37 °C. It was washed for four times. 50 μl of chromogen solution A and 50 μl of chromogen solution B was added to each well. They were mixed and incubated for 10 min at 37 °C. 50 μl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450 nm wavelength within 15 min taking the blank well as zero concentration.

Calculation

A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

3. Statistical Analysis

Data was analyzed using Statistical Package for Social Sciences (SPSS) version 20. Student *t*-test, ANOVA (Analysis of Variance), Pearson product moment and chi-square were the tools employed. Results were expressed as mean ± standard deviation and are presented in tables and significance level was set at $p < 0.05$.

4. Results

The results showed difference that was statistically significnat ($p < 0.05$) in IFN-γ(48.94 **±** 3.73 pg / ml, 46.64 ± 1.71 pg / ml, 38.76 ± 1.48 pg / ml, 26.77 ± 1.27 pg / ml, $p = 0.000$), IL-6 (15.92 ± 2.43 pg / ml, 14.10 ± 1.97 pg / ml, 11.14 ± 1.31 pg / ml, 8.66 ± 1.12 pg / ml, $p = 0.000$) IL-10 (21.06 ± 2.84 pg / ml, 18.93 ± 1.34 pg / ml, 15.08 ± 1.12 pg / ml, 10.62 ± 1.19 pg / ml, $p = 0.000$), CD4 (206.32 ± 13.50 cells / L, 235.83 ± 14.16 cells / L, 285.93 ± 8.13 cells / L,

329.10 ± 23.41 / L, p = 0.000), hepcidin (50.48 ± 3.79 ng / ml, 37.28 ± 4.00 ng / ml, 31.57 ± 3.14 ng / ml, 23.02 ± 3.88ng / ml, p = 0.000), Iron (61.40 ± 8.68 μg / dl, 65.54 ± 6.53 μg / dl, 80.85 ± 6.35 μg / dl, 100.97 ± 12.85 μg / dl, p = 0.000), TIBC (286.44 ± 18.80 μg / dl, 296.60 ± 17.36 μg / dl, 324.35 ± 23.25 μg / dl, 346.52 ± 18.16 μg / dl, p = 0.000), %TSA (21.49 ± 3.01%, 22.30 ± 2.81%, 25.13 ± 3.38%, 29.19 ± 3.74%, p = 0.000) WBC (5.41 ± 0.49 X 10^9 / L, 4.87 ± 0.27X 10^9 / L, 5.93 ± 0.65 X 10^9 / L, 5.40 ± 1.41 X 10^9 / L, p = 0.000), Neu (63.02 ± 2.74%, 60.64 ± 1.02%, 58.11 ± 1.72%,53.30 ± 3.59%, p = 0.000), Lymphocytes (22.00 ± 2.90%, 26.06 ± 1.41%, 32.94 ± 2.49%, 40.33 ± 3.57%, p = 0.000), Monocytes (11.44 ± 0.94%, 10.33 ± 0.73%, 7.04 ± 1.23%, 5.34 ± 0.87%, p = 0.000), Eosinophils (2.22 ± 0.47%, 1.16 ± 0.17%,0.92 ± 0.18%, 0.48 ± 0.18%, p = 0.000), Basophil (1.33 ± 0.26%, 1.79 ± 0.34%, 0.99 ± 0.32%, 0.55 ± 0.18%, p = 0.018), RBC (3.64 ± 0.19 X 10^{12} / L, 3.57 ± 0.46 X 10^{12} / L, 4.31 ± 0.20 X 10^{12} / L, 4.50 ± 0.17 X 10^{12} / L, p = 0.000), Hemoglobin (10.91 ± 0.56 g / dl, 10.71±1.39 g / dl, 12.91 ± 0.61 g / dl, 13.51 ± 0.54 g / dl, p = 0.000), PCV (32.72 ± 1.68%, 32.14 ± 4.16%, 38.74 ± 1.83%, 40.53 ± 1.61%, p = 0.000), MCV (78.88 ± 1.48 fl, 76.72 ± 1.33 fl, 81.60 ± 2.74 fl, 83.60 ± 1.17 fl, p = 0.000), MCH (27.52 ± 0.40 pg, 27.92 ± 0.75 pg, 29.88 ± 1.20 pg, 33.47 ± 1.17 pg, p = 0.000), MCHC (352.30 ± 0.40 g / l, 341.80 ± 0.97 g / l, 326.24 ± 18.20 g / l, 338.28 ± 27.25 g / l, p = 0.000), Platelets (140.98 ± 3.66 X 10^9 / L, 148.11 ± 6.62 X 10^9 / L, 184.57 ± 4.72 X 10^9 / L, 203.1 ± 28.26 X 10^9 / L, p = 0.000), ESR (47.55 ± 2.67 mm / hr, 38.40 ± 3.66 mm / hr, 30.13 ± 2.53 mm / hr, 21.05 ± 2.91 mm / hr, p = 0.000) when compared among PTB on Baseline, two months, four months and six months of treatment respectively.

5. Discussion

Interferon gamma has been implicated as the major cytokine that is released in tuberculosis infection. The levels of interferon gamma decreased in the PTB subgroups and were statistically significant. Decrease in interferon gamma levels were observed in this study based on the increasing duration of treatment.

The results showed that the levels of IL-6 based on the duration of treatment showed decrease that was statistically significant among pulmonary TB subgroups on two months, four months and six months compared to pulmonary TB on baseline and pulmonary TB on six months treatment showed no significant difference. This shows that treatment of tuberculosis has a decreasing effect on IL-6 which is seriously implicated in the pathogenesis of tuberculosis as the major regulatory cytokine that regulates the major hormone, hepcidin that regulates the synthesis and release of iron which is a major mechanism of the MTB survival means in the patients. Some works have revealed that when tuberculosis infection occurs, a variety of pro and anti-inflammatory cytokines are produced at disease sites and then released into circulation (Munk and Emoto, 1995; and Deveci *et al.*, 2005). IL-10 is one of the most important anti-inflammatory cytokines reported to affect multiple cell types, including macrophages, monocytes, dendritic cells,, CD4 T cells and CD 8 T cells (Moore *et al.*, 2001). IL-10 is one of the most important anti-inflammatory cytokines reported to inhibit CD4 + T cell responses by inhibiting APC function of cells infected with mycobacteria (Rojas *et al.*, 1999). The results showed that the levels of IL-10 were significantly higher in pulmonary TB patients compared to healthy subjects ($p < 0.05$). The findings are similar with previous studies that have shown higher levels of IL-10 in the active pulmonary TB group than in the control group (Olobo *et al.*, 2001; Bonecini-Almeide *et al.*, 2004; and Jung *et al.*, 2003). IL-10 can be found in the serum, plasma and bronchoalveolar larvage fluid of active pulmonary TB patients and may contribute to the anergy and failure of lymphocytes to proliferate in response to pulmonary TB (Huard *et al.*, 2003; and Redford *et al.*, 2011). It is the balance between the inflammatory and protective immune response that determines the outcome of tuberculosis infection (Sharma and Bose, 2001).

The CD4 levels based on duration of treatment was higher among pulmonary TB after six months treatment than pulmonary TB on baseline. This implies that there was improvement in the immunity of pulmonary TB patients due to the treatment especially when the duration of treatment was longer.

Hepcidin is the main hormone that regulates the synthesis and release of iron in the body. Hepcidin is an acute phase reactant peptide that is the central regulator of iron homeostasis, and its expression is modulated by several factors, including body iron status and hypoxia (Piperno *et al.*, 2009). Similarly, infections and inflammation may stimulate hepcidin expression by hepatocytes, a process that is mediated via proinflammatory cytokines, usually IL-6 , and signaling through the STAT-3 pathway (Armitage *et al.*, 2011; and Ganz and Nemeth, 2012). Hepcidin leads the process of Anemia of Chronic Disease (ACD) by causing iron to be diverted from the circulation and sequestered within cells of the reticuloendothelial system and by inhibiting duodenal absorption of iron. Thus, as a consequence of inflammation, hepcidin restricts the availability of iron for incorporation into erythroid progenitor cells (Weiss and Goodnough, 2005). The increase in hepcdin in these patients may be associated with the anemia observed in the body and this may be a

Table 1: Mean ± SD values of interferon-gamma, IL-6, IL-10, CD4, hepcidin, iron, and some hematological parameters of PTB subjects based on duration of treatment

Parameters	Baseline	2 Months	4 Months	6 Months	*F*-value	*p*-value
IFN-γ (pg/ml)	48.94 ±± 3.73	46.64 ±± 1.71	38.76 ±± 1.48	26.77 ± 1.27	1073.320	0.000*
IL-6 (pg/ml)	15.92 ± 2.43	14.10 ± 1.97	11.14 ± 1.31	8.66 ± 1.12	154.677	0.000*
IL-10 (pg/ml)	21.06 ± 2.84	18.93 ± 1.34	15.08 ± 1.12	10.62 ± 1.19	376.643	0.000*
CD4 (cells/l)	206.32 ± 13.50	235.83 ± 14.16	285.93 ± 8.13	329.10 ± 23.41	172.600	0.000*
Hepcidin (ng/ml)	50.48 ± 3.79	37.28 ± 4.00	31.57 ± 3.14	23.02 ± 3.88	374.510	0.000*
Iron (μg/dl)	61.40 ± 8.68	65.54 ± 6.53	80.85 ± 6.35	100.97 ± 12.85	67.946	0.000*
TIBC (μg/dl)	286.44 ± 18.80	296.60 ± 17.36	324.35 ± 23.25	346.52 ± 18.16	58.456	0.000*
%TSA (%)	21.49 ± 3.01	22.30 ± 2.81	25.13 ± 3.38	29.19 ± 3.74	14.181	0.000*
WBC (X 10^9/L)	5.41 ± 0.49	4.87 ± 0.27	5.93 ± 0.65	5.40 ± 1.41	23.481	0.000*
Neu (%)	63.02 ± 2.74	60.64 ± 1.02	58.11 ± 1.72	53.30 ± 3.59	134.183	0.000*
Lym(%)	22.00 ± 2.90	26.06 ± 1.41	32.94 ± 2.49	40.33 ± 3.57	42.762	0.000*
Mon (%)	11.44 ± 0.94	10.33 ± 0.73	7.04 ± 1.23	5.34 ± 0.87	53.833	0.000*
Eos (%)	2.22 ± 0.47	1.16 ± 0.17	0.92 ± 0.18	0.48 ± 0.18	9.443	0.000*
Bas (%)	1.33 ± 0.26	1.79 ± 0.34	0.99 ± 0.32	0.55 ± 0.18	3.404	0.018*
RBC (X 10^{12}/L)	3.64 ± 0.19	3.57 ± 0.46	4.31 ± 0.20	4.50 ± 0.17	105.259	0.000*
Hb (g/dl)	10.91 ± 0.56	10.71 ± 1.39	12.91 ± 0.61	13.51 ± 0.54	84.571	0.000*
PCV (%)	32.72 ± 1.68	32.14 ± 4.16	38.74 ± 1.83	40.53 ± 1.61	103.884	0.000*
MCV (fl)	78.88 ± 1.48	76.72 ± 1.33	81.60 ± 2.74	83.60 ± 1.17	116.976	0.000*
MCH (pg)	27.52 ± 0.40	27.92 ± 0.75	29.88 ± 1.20	33.47 ± 1.17	129.674	0.000*
MCHC (g/l)	352.30 ± 0.40	341.80 ± 0.97	326.24 ± 18.20	338.28 ± 27.25	39.402	0.000*
Plt (X 10^9/L)	140.98 ± 3.66	148.11 ± 6.62	184.57 ± 4.72	203.1 ± 28.26	165.842	0.000*
ESR (mm/hr)	47.55 ± 2.67	38.40 ± 3.66	30.13 ± 2.53	21.05 ± 2.91	187.950	0.000*

Note: Significant level – * $p < 0.05$; and ns – Not significant ($p > 0.05$).

mechanism the body uses to counteract the anemia seen in them by challenging the body to produce more iron to couple with globin to form hemoglobin to transport oxygen needed in the metabolism of the body. In patients with tuberculosis, higher hepcidin concentrations were strongly associated with more severe anemia. Since hepcidin has a well described, central role in ACD as opined by Weiss and Goodnough (2005), in which its expression is upregulated predominantly by IL-6 in response to infections such as tuberculosis (Armitage *et al.*, 2011),

The study revealed no significant decrease in iron in pulmonary TB on baseline and pulmonary TB on two months of treatment but increased significantly from four months and six months of treatment. The study shows that duration of treatment has no effect on the level of iron in pulmonary TB on baseline and two months of treatment. Distortions in iron availability are common in infectious diseases and most of these alterations may be associated to actions of the iron-regulatory homone hepcidin (Ganz, 2011). Hepcidin

breaks the major cellular iron exporter ferroportin resulting to decreased iron absorption in the intestine and iron retention in monocytes and macrophages and the spleen (Nemeth *et al.*, 2004). The duration of treatment among pulmonary TB subgroups only has significant increase from four months and six months of treatment, the effect on iron should be expected from four months to six months. The first two months will be used to adjust the system of the body to the treatment and will increase but will not be significant.

The TIBC of pulmonary TB on baseline was lower than four months and six months on treatment. The pulmonary TB on six months of treatment has higher level of TIBC than two months and four months on treatment.

The results showed decrease that was statistically significant in red blood cell, hemoglobin and packed cell volume which may be the cause of anemia usually seen in pulmonary TB patients. These parameters improved with increased duration of treatment. The possible mechanisms for the development of anemia during pulmonary TB infection may be due to nutritional insufficiency, impaired iron utilization, malabsorption, bone marrow granuloma and shortened duration of RBC survival (Berkowitz, 1991). Weiss (2002), Mean (2003) and Nemeth *et al.*(2004) explained the mechanism causing anemia in pulmonary TB, saying that the invasion of bacteria leads to activation of T-lymphocyte and macrophages, which induce the production of the cytokines like interferon gamma(IFN-γ) and IL-6 which with their products will divert iron into iron stores in the reticuloendothelial system resulting in decreased iron concentrations in the plasma thus limiting its availability to red cells for hemoglobin synthesis, inhibition of erythroid progenitor cell proliferation and inappropriate production and activity of erythropoietin which may lead to anemia supported response of the bone marrow to anemia.

6. Conclusion

The study shows that interferon gamma, IL-6, IL-10 and hepcidin are some of the biomarkers in the pathogenesis of pulmonary TB. The cytokines and hepcidin can be used as prognostic and diagnostic markers as their levels decreased with increased duration of treatment of the patients.

The study has shown wide variations in the hemtological indices studied. The red blood cell, packed cell volume and hemoglobin were suppressed but improved win the course of treatment. Anemia is a major factor causing morbidity and mortality in the patients in pulmonary TB patients. This will help the physicians and all health care providers handling pulmonary TB patients in tackling the challenges of drug failure and enlighten the world on the level of improvement associated to the duration of treatment that are expected to occur in the patients.

The hemtological parameters like hemoglobin, RBC and PCV increased significantly with increased duration of treatment showing improvement in health status of the patients and monocytes decreased significantly in pulmonary TB patients.

Conflicts of interest

We declared no conflicts of interest from anyone.

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